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13. ABSTRACT *(Maximum 200)*

Vaccines have significantly improved the quality of human life in the past century. However, the world-wide upsurge in recent occurrences of serious infectious diseases, including plague and anthrax, in concert with the increased mobility of Americans has raised concerns about the adequacy of currently available methods to immunize against these diseases. The goal of this proposal is to develop transgenic plants for the production of oral food-based delivery of new recombinant vaccines against the plague and anthrax. The cDNAs of *Bacillus athracis* protective antigen (PA) and the *Yersinia pestis* V and F1 antigens have been regenerated by polymerase chain reaction, cloned and sequenced. Each antigen has been cloned into the appropriate plant transformation/expression vectors and transformed into tobacco cells. Transgenic tobacco seedlings expressing *B. anthracis* PA antigen and the *Y. pestis* V antigen have been propagated. The PA and V antigens synthesized in transgenic tobacco comigrate on polyacrylamide gels with bacterial synthesized PA and V, respectively. This indicates the fidelity of synthesis of these protective antigens in plants. Our successful synthesis of these antigens in transgenic plants is the initial step in the development of plant-based vaccines against the plague and anthrax.

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Frank R. Ortiz 9/12/96

PI - Signature Date

FINAL PHASE I REPORT

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V. INTRODUCTION

Genetically engineered plants have significant promise to revolutionize medicine through the development of a cost-effective production system for edible vaccines. The major goal of the Phase I contract was to generate transgenic plant material expressing three different antigens: the *Bacillus anthracis* protective antigen (PA), the *Yersinia pestis* V antigen modified to contain a His_6 epitope to facilitate affinity purification (His-Tag V), and the *Y. pestis* F1 antigen (F1), with potential for anthrax and bubonic plague vaccines, respectively. We will briefly introduce the disease and the rationale for selection of targeted antigens prior to highlighting our Phase I results. The negotiated revision of Phase I objectives and the subsequent lag time in obtaining the necessary starting material for cloning of the *Y. pestis* antigens resulted in a delay in initiation of the plague-related aspects of the research. To account for the delayed start, we requested (and received) a 4 month no-cost extension. Phase I project dates are 1 November 1995 to 14 August 1996.

Current vaccines against *Y. pestis*. *Y. pestis* is the causative agent of bubonic plague in humans and other mammals and is endemic in regions of North and South America, Africa, and Asia (Leary et al., 1995). *Y. pestis* is one of the most virulent bacterial pathogens with the ability to infect an individual with only 100 bacteria. The major symptom of bubonic plague is the formation of the bubo, a massive inflammatory reaction in the lymph nodes, near the site of infection. This form of infection is commonly spread through a flea bite. However, the severe form of pneumonic plague can be transmitted through the air and can lead to death within 3 days (Cornelis, 1992). There are several effective vaccines which consist of attenuated or killed whole-cells of *Y. pestis* which have been developed and used for bubonic plague. However, these vaccines are not effective against pneumonic plague which infect the respiratory and intestinal regions (mucosal surfaces) (McGhee et al., 1993). Therefore, there is an urgent need for effective vaccines which will induce the mucosal immunity system against this highly lethal human pathogen.

Characterization of virulence factor genes V and F1. The highly virulent nature of *Y. pestis* is due to a diverse array of factors which are encoded on the chromosome and three endogenous plasmids (Sodeinde and Goguen, 1988; Cornelis, 1992). The major virulence factors are the Lcr genes, fibrinolysis and the capsule. A *Y. pestis* mutant with a deletion of one of the Lcr genes, LcrV (V antigen) gene is no longer virulent (Bergman, et al., 1991). The function of the 38 kDa-V antigen has not been clearly defined, but has been proposed to be involved in immunosuppression by decreasing cytokine synthesis (Nakajima and Brubaker, 1993). Rabbit antisera raised against purified V antigen (Une and Brubaker., 1984) and a recombinant V antigen protein (protein A-V antigen; Motin et al., 1994) promoted passive immunity in mice. Recently, Leary and coworkers (1995) have shown that *Escherichia coli*-synthesized V antigen administered through intramuscular injection actively immunized mice against plague (Leary et al., 1995).

The *caf*1 gene of *Y. pestis* that encodes the 17.5 kDa capsule protein, a major component of the current whole cell vaccines (Chen et al., 1976), is another antigen with potential use as an edible vaccine. The F1 antigen is a highly antigenic protein as evidenced by the high titers of antibodies to F1 antigen found in animals naturally infected with *Y. pestis* (Shepherd et al., 1986) and the active immunity to plague induced by intramuscular immunization of BALB/c mice with F1 purified from *E. coli*. When originally administered orally, the F1 antigen did not provide protection against the plague, but only provided protection in mice when administered interperitoneally (Thomas et al., 1992). The failure of oral administration of F1 to provide protection in these original experiment was eventually overcome when mice were orally vaccinated with recombinant *Salmonella typhimurium* expressing F1 antigen (Oyston et al., 1995). *S. typhimurium* is capable of colonizing the mucosal tissue in the gut and therefore may induce mucosal immunity to F1 antigen through association with the Peyer's patches (Cardenas and Clements, 1992). Therefore, if a more effective system of antigen delivery can be developed, F1 antigen will be an excellent candidate as an oral vaccine for the plague.

Current vaccines against anthrax. *Bacillus anthracis* is the bacterial pathogen responsible for anthrax disease which occurs in a variety of warm-blooded animal species including humans (Ezzel et al., 1993). It can be separated into two forms - cutaneous and septicemic. Although the cutaneous form in humans is often the result of contact with infected animals, the septicemic form can develop from a variety of initial infection sites including pulmonary infections. Both the rapid rate of linear transmission through and between affected species as well as the severity of the disease (splenomegaly, respiratory distress, circulatory collapse leading to extreme hypoxia and eventually death, sometimes within 48 hrs of infection) have made this bacterium an important target for vaccine development dating from the time of Pasteur (Vallery-Radot, 1960). Pasteur's initial anthrax vaccine was based on low temperature cultivation of wild type *B. anthracis*. This treatment resulted in the loss of a temperature sensitive plasmid encoding the protective protein antigen (Mikesell et al., 1983). Subsequent vaccines were improved in terms of delayed dissemination and stability by incorporating saponin along with spore suspensions from a non-encapsulated, toxigenic strain (Carbazoo vaccine). The current vaccine for humans (USA and Britain) consists of the *B. anthracis* 83 kDa protective antigen (PA) bound to aluminum salts and injected intramuscularly (Brackman, et al., 1962; Puziss and Wright, 1963). Although there are other virulence factors (eg. edema and lethal factors, Stepanov, 1991) defined for *B. anthracis*, immunity is induced by vaccines containing the 83 kDa protective antigen in humans.

Current strategies in the development of oral vaccines. In order to effectively protect individuals from infection with enteric, respiratory and genitourinary bacterial and virus pathogens, a vaccine must stimulate the mucosal immune system which is quite distinct from humoral immunity induced by systemic exposure. For a vaccine to be effective in stimulating mucosal immunity, the antigen must first be stable in the gut and then be able to penetrate the epithelial cells to facilitate absorption by M cells. For these reasons it has been very difficult to

develop more effective vaccines which will stimulate mucosal immunity. With the majority of vaccines tested, multiple administrations of large doses of vaccine are required to stimulate a moderate level of mucosal immunity in the gut and respiratory tract because of the antigens inefficiency of absorption to M cells. One of the very few exceptions has been the vaccine using the nontoxic cholera toxin binding subunit (CTB) which has been shown to stimulate strong mucosal immunity in both animals and humans when administered orally (Quiding et al., 1991; Holmgren et al., 1994). The CTB subunit also facilitates mucosal delivery and antigenicity of other antigens when administered as an adjuvant or when present as a fusion peptide (Shalaby, 1995).

E. coli heat-labile enterotoxin binding subunit (LTB), a homologous protein to CTB, is another example of a proven stimulator of mucosal immunity when administer to mice orally. LTB synthesized in transgenic tobacco and potato has recently been shown to stimulate both mucosal and humoral immunity responses in animals (Haq et al., 1995) and currently is being tested in humans (Steinberg, 1996). The results of the Phase I studies and other recent reports on transgenic plant-based strategies for edible vaccines (see Moffat, 1995), strengthen our strategy to test these plant-synthesized antigens for immunogenicity, protection, and tomato-based oral delivery in Phase II.

VI. EXPERIMENTAL METHODS AND RESULTS

A. Materials

Unless specified chemicals were purchased from Boheringer Mannheim, Sigma Chemical Co., Aldridge Chemical Co., Fisher Scientific Co., VWR and Baxter Scientific Products. Restriction enzymes, nucleotides and molecular biology kits were obtained from New England BioLabs, Boehringer Mannheim and Gibco-BRL and used according to the manufacturers recommendation. Antibodies were purchased from BioRad, Sigma Chemical Co. and CloneTech Inc. and used according to the manufacturers recommendation.

Plasmid Clones *B. anthracis* protective antigen plasmid clone pYS5 (GenBank Accession #M22589) was a gift of Dr. S.H. Leppla (National Institute of Health, Bethesda, Maryland). *Y. pestis* His-Tag V antigen plasmid clone, pET15b::*Icr*V (GenBank Accession # M26405) and F1 antigen clone, pBT322::*caf* (GenBank Accession #X61996) were gifts of Dr. G.P. Andrews (Bacteriology Division, USAMRIID, Fort Detrick, Maryland).

B. Methods

DNA isolation. Plasmid DNA used for polymerase chain reaction, PCR, cloning and restriction analysis was isolated according to the method described by Maniatis et al., 1989. DNA fragments smaller than 100 bp were purified on 5% polyacrylamide, TBE (0.089 M Tris-borate, 0.089 M EDTA pH 8.0) gels. The DNA band was cut out of the gel after staining with 0.5 μg/ml ethidium bromide and visualization. The gel was crushed with a glass rod in the presence of gel soaking buffer (1% SDS, 10 mM Tris pH 7.5, 0.5 mM EDTA) and incubated at 37°C overnight. The gel debris was pelleted at room temperature at 14,000 x G in a microcentrifuge for 10 min. The DNA fragment was precipitated overnight at room temperature in the presence of 0.3 M sodium acetate pH 5.5 and 3 times the volume of 100% ethanol. The resulting pellet was washed with 75% ethanol, dried and resuspended in TE. DNA fragments larger than 100 bp were run on agarose TBE gels and purified by GeneClean (Bio101 Inc.) according to the manufacturers recommendation.

Sequence analysis. Plasmid DNA prepared for sequence analysis was isolated according to the recommended methods of the University of Virginia Biomolecular Research Facility (Box 441, Jordan Hall, University of Virginia, Charlottesville, VA 22908). Bacterial cultures were grown overnight at 37°C in Terrific Broth with ampicillin (100 μg/ml). One and a half mls of culture media was pelleted at room temperature at 12,000 x G followed by resuspension in 200 ul of GTE (50 mM glucose, 25 mM Tris-HCl pH 8.0). Three hundred μl of freshly prepared denaturing buffer (0.2N NaOH/1% SDS) was added and incubated on ice for 5 min The solution was neutralized by the addition of 300 ul of 3.0 M potassium acetate pH 4.8. The cellular debris was pelleted at room temperature and the supernatant transferred to a new tube. RNase A (Sigma) was added to a final concentration of 20 μg/ml and incubated at 37°C. After 20 min the solution was extracted twice with 400 μl of chloroform. The phases were separated by centrifugation for 1 min at room temperature in a microcentrifuge. The aqueous phase was collected and DNA precipitated by the addition of equal volume of 100% isopropanol and placed in a microcentrifuge for 10 min at 14,000 x G. The plasmid DNA pellet was dissolved in 32 μl of sterilzed water and subsequently precipitated by the addition of 8.0 μl of 4 M NaCl and 40 μl of 13% PEG8000 (Sigma) and incubation on ice for 20 min. The plasmid DNA was pelleted at 4°C for 15 min in a fixed angle microcentrifuge. The resulting pellet was washed in 500 μl of 70% ethanol, dried and resuspended in 20 μl of sterilized deionized water. For sequence analysis five hundred ng of DNA was sent to the University of Virginia Biomolecular Facility (Charlottesvill, VA).

Transient expression of antigens in tobacco cell-suspension cultures. In order to rapidly assess the ability of tobacco to express a foreign protein, we have devised a system by which tobacco suspension-cultured cells are transformed with *Agrobacterium tumefaciens* containing a binary vector which harbors the gene of interest. Tobacco (*Nicotiana tabacum* cv BY-2) cells were grown in Murashige and Skoog (MS; 1962) media containing 0.2 μg/ml 2,4-D with shaking at

135 rpm and subcultured every week with a 2% inoculum. Three days after subculture, the cell suspension culture was inoculated at the rate of 1.33 x 10^9 cells/ ml culture with a 24-hr culture of *Agrobacterium tumefaciens* which was induced for 8 hr by the addition of 50 μM 3',5'-dimethoxy-4'-hydroxyacetophenone (Acros Organics, Pittsburgh, PA) to increase the efficiency of transformation during cocultivation. Following cocultivation of the BY-2 cells with *Agrobacteriua* for 12 hours, expression of the introduced gene was induced by the addition of 0.1 μg/ml cellulase (*Trichoderma viride*, Sigma Chemical Co., St. Louis, MO). After an additional 12 hours of cocultivation, the cells of the suspension culture were allowed to settle by gravity and washed 3 times with fresh MS media. The cells were then centrifuged in a clinical centrifuge and processed for protein isolation (see below). To monitor the efficiency of transformation, BY-2 cells were also cocultivated with an *Agrobacterium* strain containing a gene construct consisting of the MeGA promoter fused to the *uidA* gene of *Escherichia coli.* This gene, when expressed, gives rise to the β-glucuronidase protein whose presence in the transformed cells was monitored calorimetrically according to the method of Jefferson (1987) after the addition of the substrate 5-bromo-4-chloro-3-indolyl glucuronide (Gold BioTechnologies, Inc., St. Louis, MO). By using this construct as a control, it was determined that approximately 30% of the cells in the suspension culture were transformed.

Generation and propagation of transgenic tobacco. *Agrobacterium*-mediated transformation of tobacco leaf disks was performed by the method of Horsch et al. (1984). The appropriate engineered expression vectors were introduced into *A. tumefaciens* strain LBA4404 (Hoekman et al., 1983). Using a sterile paper punch small leaf disks were excised from axenically-grown tobacco seedlings, dipped into the bacterial suspension culture, co-cultivated for 24 hours, and transferred to soft MS (Murashige and Skoog, 1962) agar plates containing carbenicillin (500 μg/ml), kanamycin (100 μg/ml) and hormones which induce shoot and leaf formation. After incubation in a plant growth incubator for 3-4 weeks, the shoots were transferred to root-initiation medium. Transgenic plants were transferred to the greenhouse 10-15 weeks after the initial transformation.

Isolation of total leaf or BY-2 culture cell protein Total leaf or total BY-2 culture cell proteins were extracted with 1X SDS sample loading buffer (60 mM Tris-HCl, pH 8.0, 60 mM DTT, 2% SDS, 15% sucrose) by grinding tissues or cells in a ground-glass homogenizer. Samples were boiled for 5 minutes, and insoluble debris was removed by centrifugation in a micro centrifuge (12,000 x g) for 5 min Protein concentration was determined with Coomassie Blue dot blot assay (Ghosh et al., 1988) and Pierce plus protein assay (Pierce Co.) systems.

Gel electrophoresis and Immunoblotting. Proteins (approximately 5 to 40 μg per lane) were separated on 12.5% SDS-polyacrylamide gels in the buffer system of Laemmli (1970) and blotted to membrane Optitran (Schleicher & Schuell, Keene, NH). After transfer, western blots were stained with 0.2% Ponceau S (Sigma, St. Louis, MO) in water to check transfer. Nonspecific protein binding sites were blocked with blocking buffer (20 mM Tris-HCl, pH 7.5,

0.1% Tween 20, 5% milk). Blots were incubated with antiserum in antibody buffer (20 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 2.5% milk) overnight .In some experiments, primary antibodies were prewashed for 7 hr with a membrane bound with total leaf proteins from BIB-Kan plants to reduced the nonspecific cross-reaction from leaf proteins. Blots were washed three times with wash buffer (20 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 1% milk, 0.5% BSA) and incubated for 1 hr in antibody buffer containing alkaline phosphatase-conjugated goat anti-mouse IgG (1:20,000 dilution, Clontech, Polo Alto, CA). Blots were washed three times with antibody buffer, incubated with Chemiluminescent substrate (CSPD) in assay buffer (100 mM diethanolamine, pH 10.0, 1 mM $MgCl_2$), and exposed to X-ray film.

C. Results

Phase I, Objective 1. Generate plant transformation/expression vector encoding protective antigen (PA) of *Bacillus anthracis*, the agent of anthrax.

Cloning of the MeGA:PA gene.

The complete sequence for the protective antigen, PA, from *Bacillus anthracis* has been published (Welkos et al., 1988) and *E. coli* containing a PA-encoding plasmid pYS5 was obtained from Dr. S.H. Leppla (Laboratory of Microbial Ecology, NIH/NIDR). The *B. anthracis* PA protein has a bacterial amino terminal signal peptide. To express the mature PA protein in plants the bacterial signal peptide sequence had to be removed. A PCR amplification strategy was initiated to obtain a leaderless PA clone and a promotor spacer region for subcloning the 2.2 kb gene into the plant transformation/expression vector. This required to two separate sets of PCR oligo primers and reactions. The first PCR reaction utilized primers PA1 And PA2 (Figure 1). Primer PA1 (5' end) was designed to include an 8 bp spacer sequence and the ATG start codon. Primer PA3 (3' end) was used to introduce *Sac*I and *Eco*RI restriction sites for ease of cloning. The final PCR product generated was of the expected size of 2.2 kb. This modified PA cDNA was inserted downstream of CropTech's proprietary inducible promoter, now termed the MeGA™ system (Fig. 1), using the *XbaI* site. The entire MeGA™-PA cDNA fragment was then cloned into the *Kpn*I/*Sac*I site of the plant expression vector, pBIB-Kan.

Sequence analysis of MeGA:PA.

A series of DNA primers were utilized to generate the sequence of the full length 83 kDa PA protein (2.2 kb). The universal primers T7 and T3 were used to sequence the first 600 bases at the 5' and 3' ends of the gene, respectively. Three internal PA specific 18 base primers were generated (Gibco BRL Life Technologies) in order to sequence the remaining internal region:
5' TGAGATGTTTGTTGATCG 3' (bp region 2881-3481)
5' TAGTGAATGATCAATTGC 3' (2895-2295)
5' ATTCCATCATTGTCACGG 3' (1839-2439) . The sequence of the MeGA™ promoter, junction between the MeGA™ promoter and PA and the leaderless PA gene had the expected sequence.

Phase I, Objective 2. Generate plant transformation/expression vectors encoding His-Tag V and F1 antigens of *Yersinia pestis*, the agent of plague.

The cloning of the MeGA™:His-TagV antigen.

The complete sequence of the V antigen operon of *Y. pestis* which includes the *Lcr*V protein (V antigen) has been determined (Price et al, 1989). *E.coli* containing the V antigen sequence in plasmid pET15b::*Lcr*V was received from Dr. Gerard P. Andrews, Bacteriology Division, USAMRIID on January 25, 1996. In this vector, the V antigen sequence is cloned into the *Nde*I/*Bam*HI site of pET15b (Novagen Inc., Madison, WI) which provided an in-frame His_6 epitope fused to the amino terminus of V (Figure 2). From restriction analysis of pET15B::*Lcr*V plasmid DNA, the resultant restriction map varied from the published restriction map of pET15b and V antigen. The map indicated that the plasmid contained more DNA than just the V antigen gene (Figure 3). To insure that only the His-tagged V antigen was cloned and expressed in plants, we regenerated the His-tagged V antigen cDNA by PCR using flanking primers T7 and CropTech's V2 (Figure 2) followed by cloning into the plant transformation/expression vector as shown Figure 2.

T7 primer and V2 primer which contains the carboxyl terminus of V flanked by a SacI site, were used to PCR amplify the his-Tag V antigen sequence with Vent DNA polymerase (Biolabs, Beverly, MA)). The expected 1 kb fragment containing the His-tagged V antigen gene was generated when analyzed by agarose gel electrophoresis. The 1.0 kb His-TagV antigen was digested with *Nco*I and blunt ended with Klenow polymerase (Boehringer Mannheim) . The modified His-Tag V antigen sequence was cloned into the pMeGA™ plasmid clone and used for sequence analysis. To generate the His-Tag V plant transformation/expression vector, the *Hind*III/*Sac*I (MeGA™:His-Tag V) was cloned into pBIB-Kan (Figure 2).

The cloning of the MeGA: F1 antigen.

The *caf*1 (F1 antigen) gene sequence of *Y. pestis* has been previously described (Galyov et al., 1991). We received *E.coli* cells containing the plasmid clone pBR322::*cafe* from Dr. Gerard P. Andrews, Bacteriology Division, USAMRIID on January 25, 1996. Restriction analysis of pBR322::*caf* showed that the entire caf operon was present in the plasmid clone (Figure 3). To insure that only the F1 antigen gene is expressed in plants, the F1 antigen cDNA was regenerated by PCR using F1-1 and F1-2 oligo-primers (Figure 4) and cloned into the plant expression vector.

Oligonucleotide primer F1-1 which consists of a BamHI site, ATG start codon, and the amino terminus of the mature F1 peptide and F1-2 primer which consists of the carboxyl terminus of the F1 peptide and flanking SacI/EcoRI sites were used to PCR the 500 bp cDNA of the F1 mature peptide (Figure 4). The PCR amplified F1 DNA fragment was digested with *Bam*HI, blunted with mung bean nuclease, digested with *Sac*I and ligated to pMeGA™. To generate the F1 plant transformation/expression vector, the *Hind*III/*Sac*I (MeGA™:F1) was cloned into the pBIB-Kan vector (Figure 4).

Sequence analysis of MeGA™:His-Tag V and MeGA™:F1.

The PCR amplified His-Tag V DNA was first cloned into the pMeGA™ plasmid. Using the T3, T7 primers and an internal primer V3 (5'ATGCATTACTGCCATGAACG3') were used for sequence analysis. A comparison with the expected DNA sequence of the MeGA™ promoter, the junction and the His-Tag V cDNA (GenBank Accession #M26405) showed that all sequences were correct. The F1 antigen cDNA generated by PCR amplification was first cloned into pMeGA™ BamHI/SacI sites (pBS(SK)II vector). The T3 and T7 primers were used to sequence the 900 bp MeGA™:F1. Sequence analysis showed that the sequence of the MeGA™ promoter, junction between MeGA™ promoter and F1 and the 500 bp coding sequence were correct (GenBAnk Accession #X61996).

Phase I, Objective 3. Introduce the PA, His-Tag™ V and F1 antigen genes into tobacco via *Agrobacterium*-mediated transformation.

Tobacco leaf disc transformation using *A. tumefaciens* harboring the antigen-containing constructs was performed as described in the Methods Section. As shown in Table 1., plant transformation/expression vectors, carrying either the PA, His-Tag V and F1 antigen gene were used to transform tobacco. Using two independently derived constructs, 42 transgenic tobacco plants have been propagated expressing PA antigen. The number of transgenic tobacco plants produced from the transformation of 650 leaf discs with MeGA™:PA construct is low in comparison to His-Tag V and F1 antigen constructs. Approximately one transgenic plant was generated from 2 leaf disc transformations when MeGA™:F1 construct was used in comparison to 30 leaf disc in the case of MeGA™:PA construct. The low number of transgenic tobacco plants expressing PA may be due to the impact of the sequence on the transformation efficiency or the early growth and development . However, once the seedlings formed, the growth and development of the PA transgenic seedlings was found to be indistinguishable from F1 transgenic seedlings.

Table 1. **Generation of transgenic tobacco seedlings.**

Clone	antigen	# of leaf discs transfected	#seedlings propagated in soil
pCT59	PA	300	12
pCT62	PA	350	30
pCT69	F1	200	110
pCT71	His-Tag V	200	98

Phase I, Objective 4. Quantify synthesis and initiate characterization of plant synthesized PA, His-Tag V and F1 antigens.

Isolation and detection of transgenic PA antigen.

Leaves from a plant transformed with MeGA:PA construct (pCT62) was harvested and induced for 8 hr prior to placement in liquid nitrogen for analysis and storage at -80 C. Frozen leaf material (0.5 g quantities) was ground in SDS sample loading buffer (1:3 tissue:buffer ratio) using a glass dounce homogenizer. Crude extracts were centrifuged twice at 14,000 X G to remove cellular debris. Aliquots (100 µl) were TCA precipitated and pellets resuspended in protein dilution buffer (150 mM NaCl, 50 mM Tris-HCl pH 7) prior to total protein concentration determination using a Coomassie Blue dot blot assay (Ghosh et al., 1988). Approximately 40 µg total protein/plant sample was separated by SDS polyacrylamide gel electrophoresis (10% acrylamide). Controls consisted of 30 ng purified PA antigen and 40 µg untransformed (UT) crude protein extract. Following separation and electrotransfer to nitrocellulose, the membrane was incubated with PA antibody (1:5000 dilution; was a gift of Dr. S.H. Leppla ,National Institute of Health, Bethesda, Maryland) that had been prewashed for 6 hr with UT extract. Membrane bound proteins were visualized following exposure to a goat anti-rabbit alkaline phosphatase secondary antibody conjugate (Clontech Chemiluminescent detection system). Figure 5 shows the production of the full length transgenic 83 kDa protein (plant 011-04) which comigrated with the bacterial synthesized PA.

Isolation and detection of transgenic His-Tag V antigen.

The detection of His-Tag V antigen from transgenic plant extracts is dependent both on the specificity and titer of the antibody to the V antigen. The monoclonal antibody to V antigen (1:20,000 dilution; gift from Dr. Gerard P Andrews Bacteriology Division, USAMRIID) was shown by western analysis to detect a minimum of 1-2 ng of V antigen (data not shown). To determine if there are any proteins present in plant extracts which cross-react with anti-V, crude

plant protein extracts from BY2 tobacco suspension culture cells and control transgenic vector only (pBIB-Kan with no transgene) plant tissue were analyzed by western analysis. As shown in Figure 6, there is an abundant 39 kDa plant protein which comigrates with the control V antigen. In order to specifically detect the presence of transgenic His-Tag V antigen, we had to first find a method to remove the endogenous cross-reacting plant protein from plant extracts. Neither differential ammonium sulfate precipitation (Figure 7) or DEAE cellulose chromatography (Figure 8) was successful at removing the endogenous cross-reacting plant protein. As mentioned previously, the V antigen was constructed with an amino His_6-epitope which specifically binds to nickel resin (Ni). When crude protein extracts from transgenic vector only tissue was bound to Ni resin, washed and eluted, the 39 kDa endogenous protein was shown not to bind to the Ni resin (Figure 9). Ni chromatography was shown to be a one step method to remove the cross-reacting endogenous protein from plant extracts.

Isolation of transgenic His-Tag V antigen: As described previously, the MeGA™:His-Tag V was transiently expressed in BY2 cells (tobacco cell suspension culture) . BY2 cell proteins from transfected and non-transfected cells were extracted and processed separately. Proteins were extracted from BY2 cells with the binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, and 500 mM NaCl) in a ground-glass homogenizer. The protein samples were passed through a 23 gauge syringe three times to shear the DNA and were spun at 48,000 X G for 20 min at 4 C. The post-centrifugation supernatant was then filtered through a 0.45 micron membrane to remove the residual particulate matters. The filtered extract was loaded to a His-bind resin Ni column (500 µl bed volume, Novagen Inc, Madison, WI). After wash the column was washed with 10 volumes of binding buffer and 6 volumes of wash buffer (20 mM Tris-HCl, pH 7.9, 60 mM imidazole, and 500 mM NaCl), the proteins bound to the column were eluted with 6 volumes of elute buffer (20 mM Tris-HCl, pH 7.9, 1000 mM imidazole, and 500 mM NaCl). Proteins from different fractions were precipitated with 10% TCA and resuspended in SDS-sample loading buffer. These samples were then analyzed on 12.5% polyacrylamide gels and western blots as described above. As shown in Figure 10, only the BY2 cells transfected with the plant transformation/expressing vector pCT71 (MeGA™:His-Tag V) had a 38 kDa protein which specifically bound to the Ni column. The 38 kDa protein represents the transgenic His-Tag V antigen synthesized in tobacco.

Detection of transgenic F1 antigen.

Two monoclonal antibodies, 3G8 and 6H3, to F1 antigen were provided by Dr. Gerard P Andrews (Bacteriology Division, USAMRIID). The detection of F1 antigen (1:500 dilution) from transgenic plant extracts is dependent on both the specificity and titer of the monclonal antibody to the F1 antigen. Both monclonal antibodies were shown not to cross-react to any endogenous plant proteins which is an indication of high specificity. The titier of both monoclonal antibodies was found to be poor. As shown in Figure 11, the 3G8 and 6H3 monoclonal antibodies to F1 detected a minimum of 500 to 1000 ng and 200 to 400 ng,

respectively. To date the low titer of F1-specific antibodies has made it very difficult to detect the transgenic F1 antigen from both transgenic leaf and BY2 cell protein extracts (Figure 12).

D. Conclusions

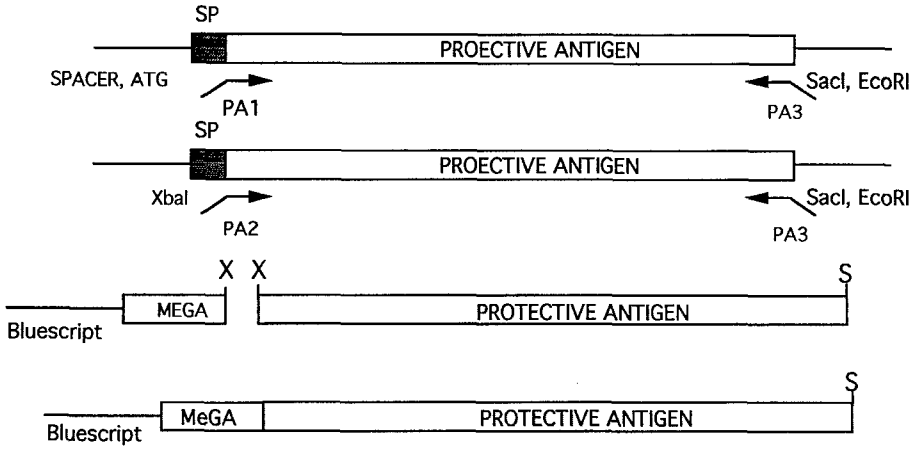
The goal of this proposal is to develop transgenic plants for the production of oral food based delivery of new recombinant vaccines against the plague and anthrax. The cDNAs of *Bacillus athracis* protective antigen (PA) and the *Yersinia pestis* V and F1 antigens have been regenerated by polymerase chain reaction, cloned and sequenced. Each antigen has been cloned into the appropriate plant transformation/expression vectors and transformed into tobacco cells. We have shown by western analysis that transgenic PA and His-Tag V antigens synthesized in plants are similar to the bacterial synthesized antigens. This is the first time these bacterial antigens have been successfully expressed in plants which is the first step in the process of testing the feasibility of producing an edible oral vaccine for bubonic plague and anthrax in plants. In order to greatly increase the potential of plant-based oral vaccines, large quantities of purified antigen must first be obtained. To perform this task transgenic plants expressing high levels of transgenic antigen must be identified. Individual transgenic plants show wide variation in levels of transgene expression due to positional effects imposed by the site of insertion. We have generated 50 to 100 transgenic plants for each of the three antigens, PA, His-Tag V and F1. The process of generating more transgenic plants is continuing. From previous experience (see Appendices), we anticipated at least 1 high antigen expressing plant from 50 transgenic plants.

The ease of purification of the transgenic antigen from plant extracts will greatly facilitate the immugenicity studies in mice. We have shown that similar to the bacterial synthesized *Y. pestis* His-Tag V antigen, the plant synthesized antigen can be purified from plant extracts in one step using Ni chromatography. As a consequence, when high expressing transgenic plants have been identified, it should be fairly easy to purify this *Y. pestis* antigen. The *B. anthracis* PA antigen does not have an epitope. As a result, it will be more difficult to purify the transgenic PA antigen from plant extracts. The high PA expressing plants will help in the development of a purification strategy for PA.

Transgenic plants and transformed tobacco cultured cells containing the F1 gene construct were produced. However, standard western immunoblot analyses of tobacco extracts did not reveal novel F1-cross-reacting proteins. We believe that this lack of detection is due to the low sensitivity of the F1 antisera. Both monoclonal antibodies 3G8 and 6H3 are 40-50 times less sensitive than the monoclonal His-Tag V antigen (see result section). Sequence analysis has shown that the MeGA™:F1 construct has the expected sequence of promote and gene. Since we have generated 110 transgenic F1 plants, when we obtain an antibody with a higher sensitivity to F1 antigen, there is no doubt that the transgenic F1 will be detected. The F1 antigen also does not have an epitope tag. Therefore, a methodology must first be developed to purify the transgenic F1 antigen from plant extracts.

In summary, the primary goal of the Phase I feasibility study was to demonstrate the ability of plants to correctly synthesize these bacterial antigens with potential as oral vaccines for anthrax and bubonic plague. We have successfully introduced genes encoding the anthrax PA antigen and the plague V and F antigens into tobacco. For the PA and V transgenes, the fidelity of the product was demonstrated based on immunodetection and electrophoretic co-migration with bacterially synthesized and purified antigen standards. F1 antigen containing plants were generated but confirmation of the level of F1 synthesis must await the development of a more sensitive immonodetection tools. Thus, the results of Phase I provide strong evidence for the promise of plant-based vaccines and most significantly provides the transformed material which will facilitate meeting our Phase II goals.

A. PCR strategy for generation of coding region for mature PA



B. Strategy for insertion of PA sequence into plant expression vector

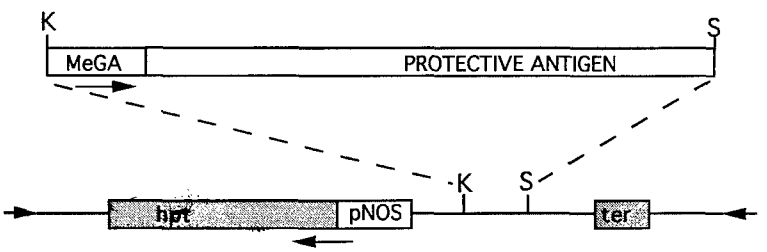


Figure 1. PCR amplification (A) and subcloning (B) strategy for recombinant protective antigen (PA). A: Arrows represent locations of 5' PCR primers PA1 (PS = 8 bp promotor spacer, ATG start codon) and PA2 (*Xba*I) and 3' PCR primer PA3 (*Sac*I, *Eco*RI). SP is the signal peptide sequence. The PA *Xba*I/*Sac*I fragment was ligated to the MeGA™ plamid (Bluescript(SK):MeGA™). B: Flanking arrows indicate the right and left border sequences that delineate the T-DNA region, and smaller arrows, the direction of transcription. MeGA™ is an inducible promotor; pNOS, a constitutive nos promotor from *Agrobacterium tumefaciens*; hpt, a hygromycin phosphotransferase I gene that confers hygromycin resistance; and ter, a polyadenylation/terminator signal. PCR-amplification primers for PA were as follows:PA1 [5'**GTAACATC**ATGGAAGTTAAACAGGAGAACCG3'], PA2 [5'GCTCTAGA**GTAACATC**ATGGAAGTTAAACAG3'], and PA3 [5'GAATTCGAGCTCTTATCCTATCTCATAGCCT 3']; restriction sites are underlined and the 8 bp spacer is in bold. Restriction enzymes: X = *Xba*I, S = *Sac*I.

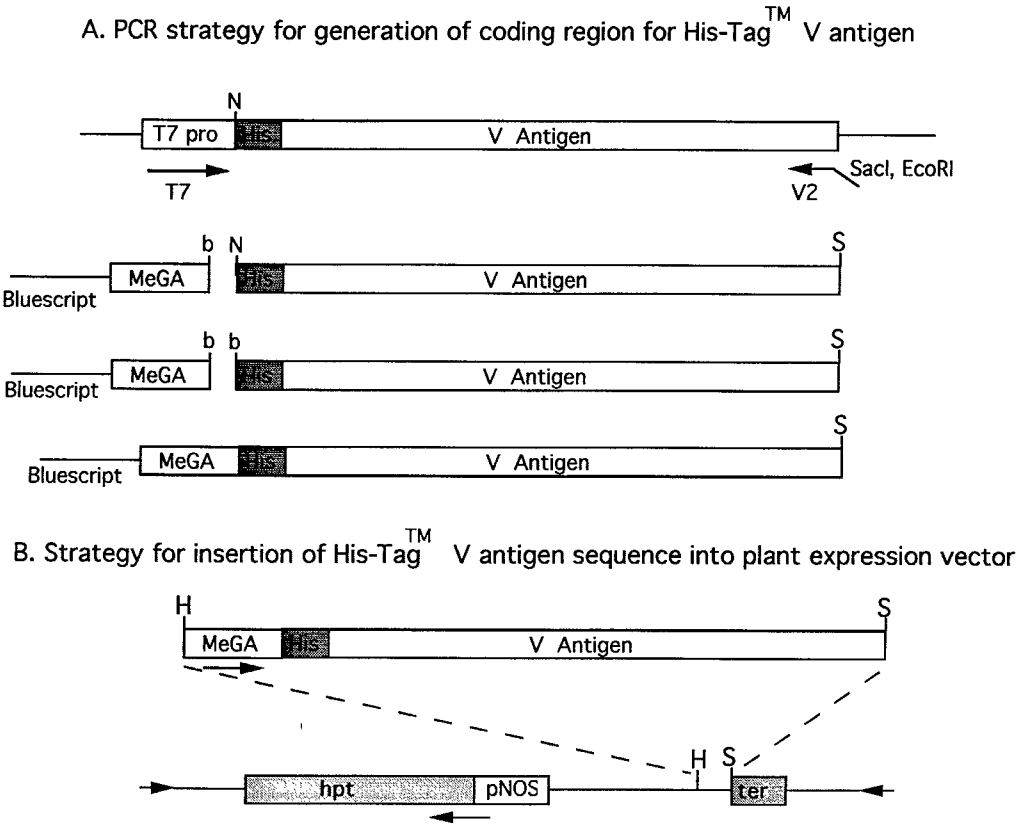
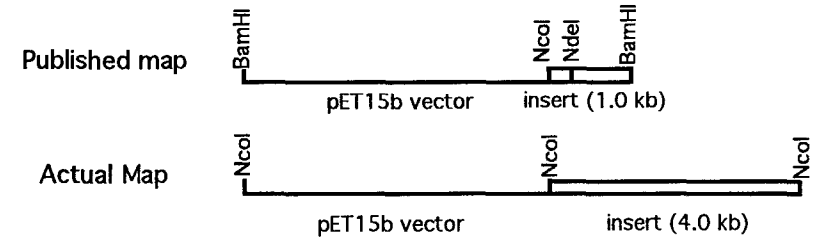


Figure 2. PCR amplification (A) and subcloning (B) strategy for recombinant His-Tag V antigen. A: Arrows represent locations of 5' PCR primer T7 3' primer V2 (*Sac*I, *Eco*RI). The His-Tag encodes a His_6 epitope fused in frame to the amino terminus of the V antigen. The blunt end ligated (b) *Nco*I site/*Sac*I His-V DNA fragment was ligated to the MeGATM plasmid (Bluescript(SK) MeGA™). The resulting MeGA™:His-Tag V plasmid was used for sequence analysis. B: The *Hind*III/*Sac*I fragment of pMeGA™:His-Tag V plasmid was ligated to pBIB-Kan. Flanking arrows indicate the right and left border sequences that delineate the T-DNA region, and smaller arrows, the direction of transcription. MeGA™ is an inducible promotor; pNOS, a constitutive nos promotor from *Agrobacterium tumefaciens*; hpt, a hygromycin phosphotransferase I gene that confers hygromycin resistance; and ter, a polyadenylation/terminator signal. PCR-amplification primers for V were as follows: T7 [5' CCCGCGAAATTAATACGACTCACTATAGGG 3'] and V2 [5'GAATTCGAGCTCTCATTTACCAGACGTGTC 3']. Restriction sites are underlined and the His-Tag is in bold. Restriction enzymes: H = *Hind*III, S = *Sac*I, N = *Nco*I.

A. Restriction map of plasmid pET15b::*Lcr*V



B. Restriction map of plasmid pBR322::*caf*

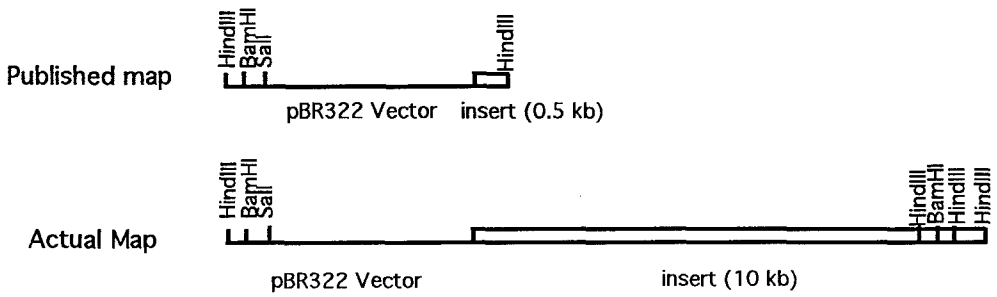
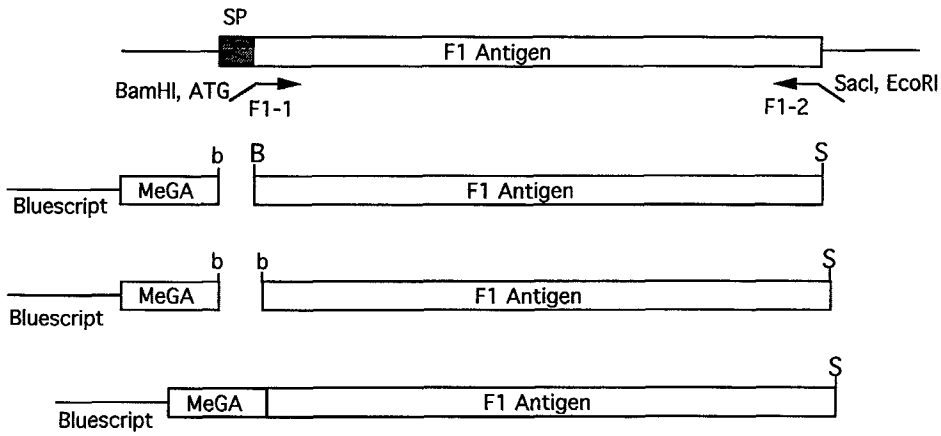


Figure 3. Restriction map of plasmid pET15b::*Lcr*V and pBR322::*caf*. A. The schematic representation of the published restriction map of *Lcr*V gene cloned into pET15b vector is as designated. The map generated by restriction analysis pET15b::*Lcr*V is shown below.. B. The schematic representation of the published map of F1 gene cloned into pBR322 is as designated. The map generated by restriction analysis of pBR322::*caf* is shown below. The sizes of the inserted DNA are denoted in kb.

A. PCR strategy for generation of coding region for mature F1



B. Strategy for insertion of F1 sequence into plant expression vector

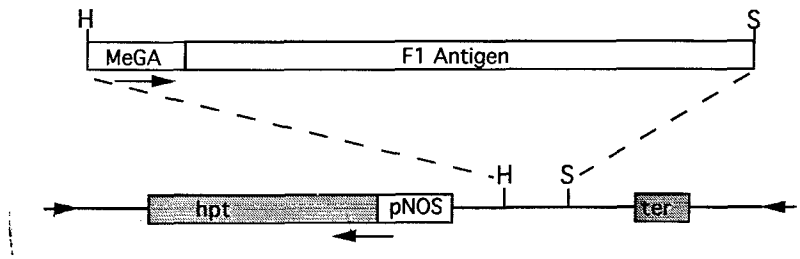


Figure 4. PCR amplification (A) and subcloning (B) strategy for recombinant F1 antigen. A: Arrows represent locations of 5' PCR primer F1-1(BamHI, ATG start codon) and 3' primer F1-2 (*Sac*I, *Eco*RI). The MeGA promoter (Bluescript:MeGA™) was blunt end ligated (b) to the BamHI site of the F1 antigen. The resulting plasmid was used for sequence analysis. B: The HindIII/SacI fragment from pMeGATMTM:F1 was ligated to pBIB-Kan vector. Flanking arrows indicate the right and left border sequences that delineate the T-DNA region, and smaller arrows, the direction of transcription. MeGA™ is an inducible promotor; pNOS, a constitutive nos promotor from *Agrobacterium tumefaciens*; hpt, a hygromycin phosphotransferase I gene that confers hygromycin resistance; and ter, a polyadenylation/terminator signal. PCR-amplification primers for F1 were as follows: F1-1 [5'CGGGATCCATGGCAGATTTAACTGCAAGC3'] and F2-2 [5'GAGAATTCGCTCTTATTGGTTAGATACGGTT 3']. Restriction sites are underlined. Restriction enzymes: H = *Hind*III, S = *Sac*I, B = *Bam*HI.

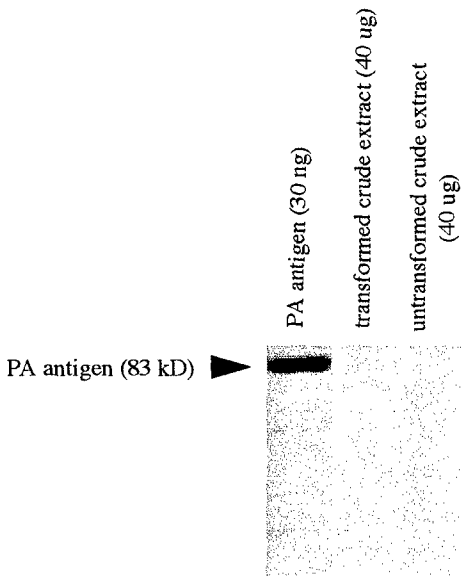


Figure 5. Western analysis of crude soluble protein extract from transgenic PA leaf material. Controls included purified PA antigen (30 ng) and untransformed tobacco crude soluble protein extract. The western blot was reacted with anti-PA antibody (1:5000 dilution) that was prewashed 6 hr with membrane bound untransformed tobacco leaf soluble proteins. The full length 83 kD PA protein is indicated by the arrow.

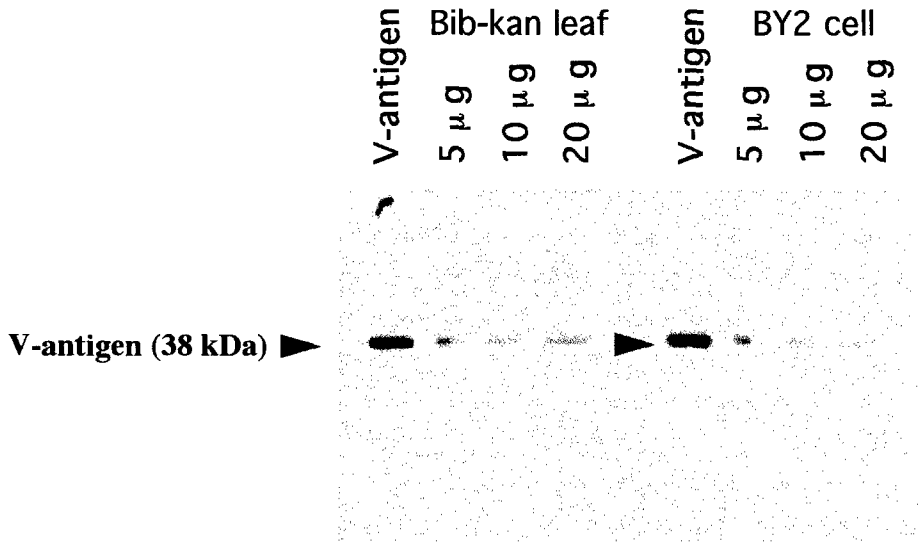


Figure 6. Western blot analysis of tobacco leaf and suspension culture cell proteins with prewashed V antibody. Five to 20 µ g of total proteins isolated from tobacco leaf (Bib-kan) and suspension culture cells (BY2) were loaded on a 12.5% polyacrylamide gel along with 20 ng of V antigen. The western blot transferred from this gel was reacted with antibodies against the V antigen (1:15,000 dilution). To reduce the nonspecific cross-reaction from proteins in tobacco samples, the antibody was prewashed 2X 5 hours with membranes bound with total leaf proteins of Bib-kan plants. The 38 kDa V-antigen is shown by the arrow. The cross-reacting polypeptide in both tobacco leaf and culture cell samples is a protein migrating similar to the V-antigen.

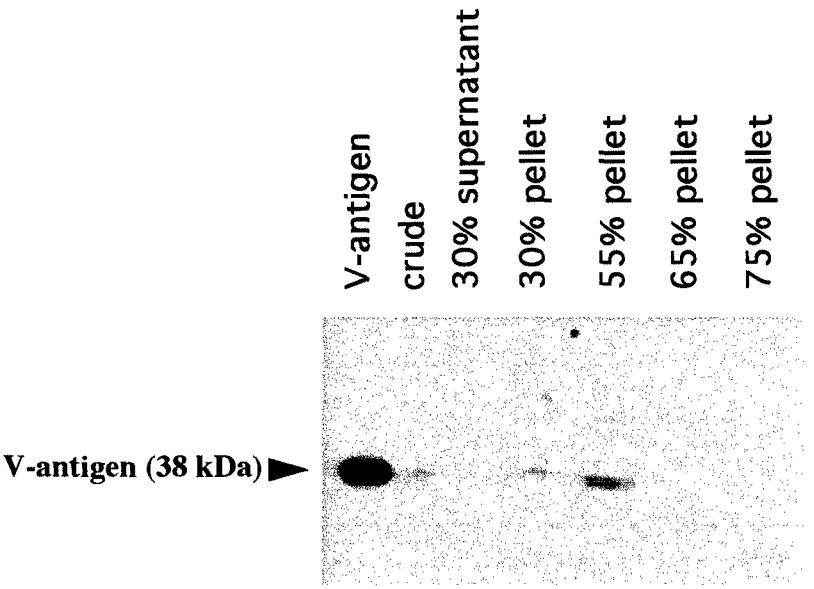


Figure 7. Western blot analysis of fractions from ammonium sulfate precipitation of total untransformed tobacco leaf proteins. Total proteins were isolated from tobacco leaf (Bib-kan) and were precipitated with ammonium sulfate sequentially to 30%, 55%, 65% and 75% saturation. The protein samples from 30% supernatuant (dialyzed), 55%, 65%, and 75% pellet fraction (resuspended and dialyzed) were analyzed on western blot with the V-antibody. The 38 kDa V-antigen is shown by the arrow. The cross-reacting polypeptide in tobacco leaf ammonium sulfate precipitation fractions is a protein migrating similar to the V-antigen.

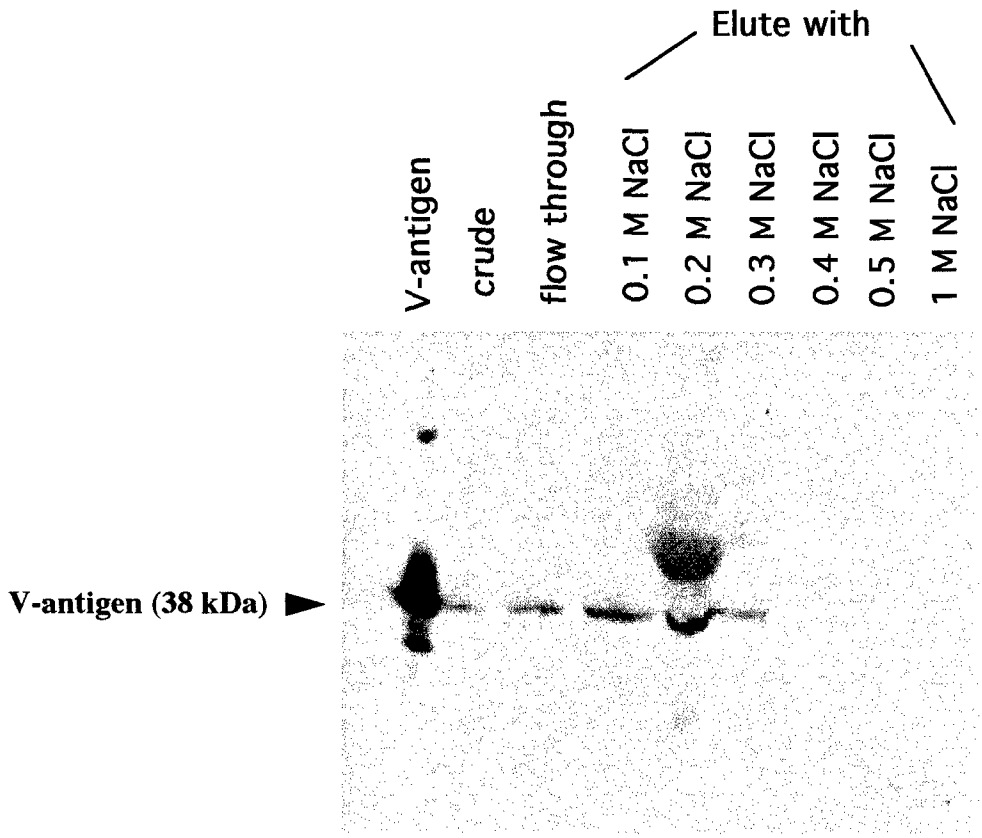


Figure 8. Western blot analysis of DEAE cellulose column fractions of total untransformed tobacco leaf protein. Total proteins were isolated from tobacco leaf (Bib-Kan) and were loaded on a DEAE cellulose ion-exchage column. After collecting the flow through factions, the columns were washed, and the bound proteins were eluded with 0.1 to 1 M NaCl. Protein samples from these fractions were analyzed on western blot with the V-antibody. The 38 kDa V-antigen is shown by the arrow. The cross-reacting polypeptide in these fractions is a protein migrating similar to the V-antigen.

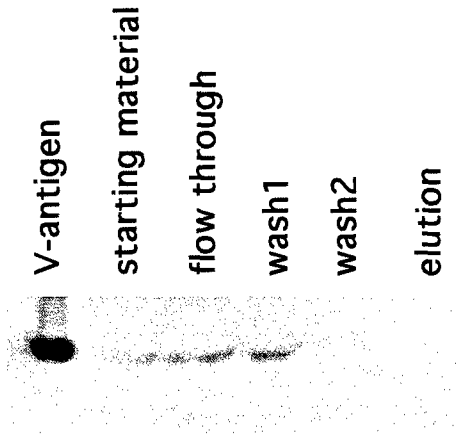


Figure 9. No cross reacting endogenous plant protein binds to the Ni column. Total leaf proteins from Bib-kan plant were extracted with the binding buffer (0.02 M Tris-HCl, pH 7.9, 5 mM imidazole, and 500 mM NaCl) . Six hundred ug of the total leaf protein was loaded to a His-bind resin Ni column (500 µl of bed volume). After wash the column with 10 volumes of binding buffer and 6 volumes of wash buffer, the proteins bound to the column were eluted with 6 volumes of elute buffer. Proteins in different fractions were precipitated with 10% TCA and then resuspended in SDS-sample buffer. These samples were loaded to a 12.5% polyacrylamide gel along with the control (20 ng V-antigen). The blot was incubated with the V-antibody (1:20,000 dilution).

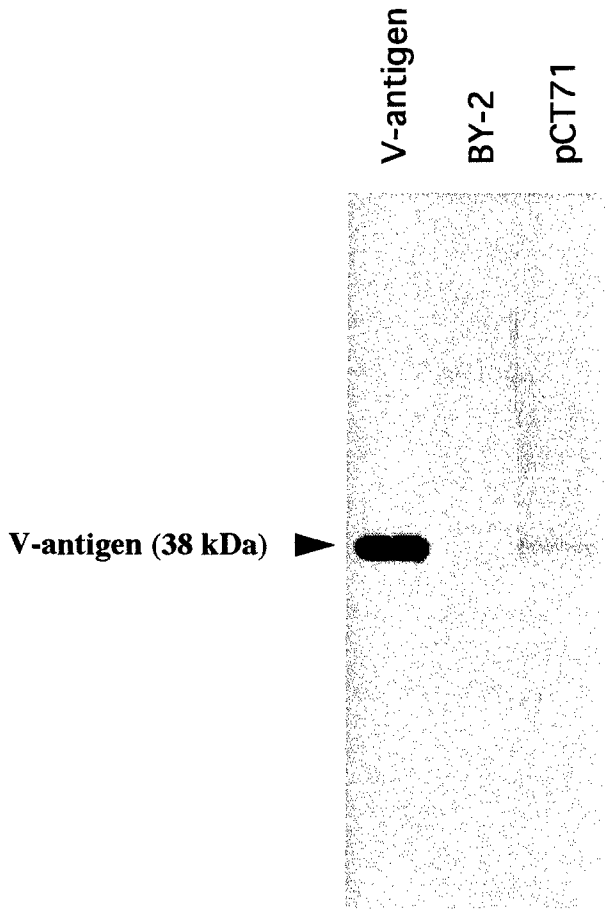


Figure 10. Western blot analysis of Ni column elution fraction of total cell protein from transformed and control BY-2 cells.
BY2 culture cells were transformed with His-tag V construct pCT71. Total cell proteins from the transformed cells were isolated with binding buffer and were applied to a His-bind resin Ni column (500 µl of bed volume). Proteins bound to the column were eluted with elute buffer and were precipitated with 10% TCA.
The protein sample was resuspended in SDS-sample buffer and was loaded to a 12.5 % polyacrylamide gel along with a BY-2 cell control sample processed in parallel. The blot was incubated with the V-antibody (1:20,000 dilution). The 38 kDa V-antigen is shown by the arrow.

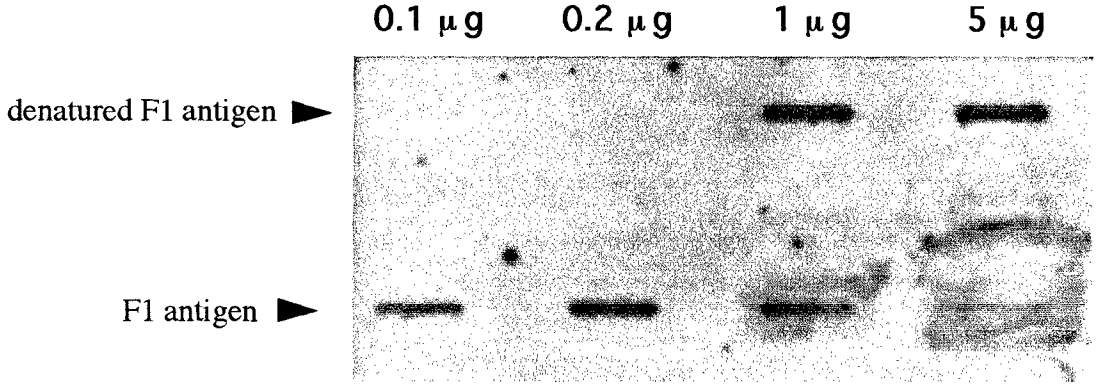


Figure 11. Slot blot analysis of bacterial F1 antigen with F1 monoclonal antibody 3G8. The purified F1 antigen (0.1 to 5 mg) was applied to a slot blot. The antigen was either loaded directly to the blot without any treatment or denatured by boiling. The slot blot was analyzed with a monoclonal antibody agains the F1 antigen (3G8, 1:500 dilution).

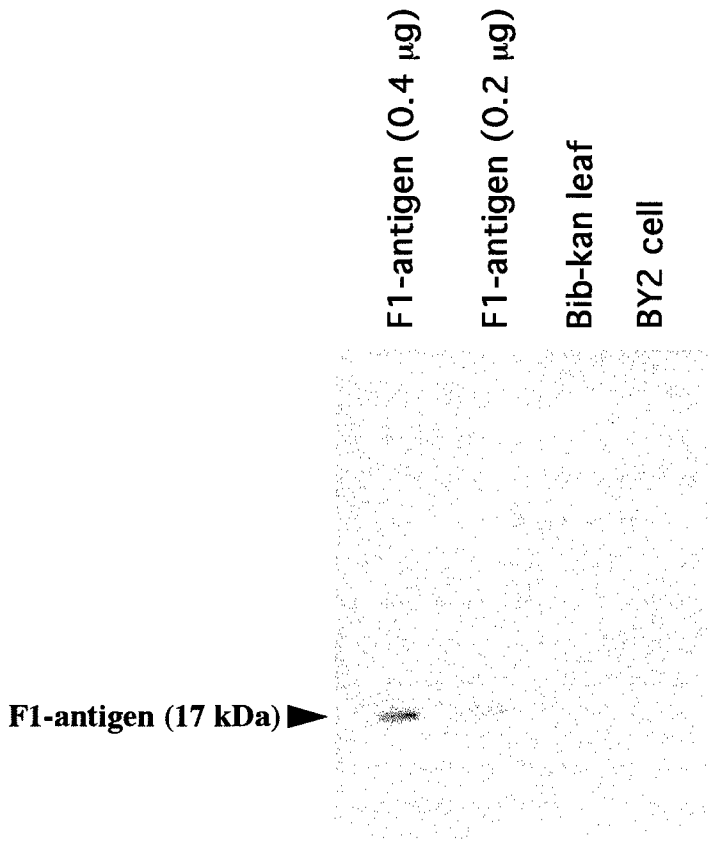


Figure 12. Western blot analysis of tobacco leaf and suspension culture cell proteins with F1 antibody. Total proteins were isolated from tobacco leaf (Bib-Kan) and suspension culture cells (BY2). Twenty m g of total protein were loaded on a 12.5% polyacrylamide gel along with 0.2 to 0.4 m g of V antigen. The western blot was reacted with a monoclonal antibody against the F1 antigen (6H3, 1:500 dilution).
The 17 kDa F1-antigen is shown by the arrow.
No cross-reacting band was detected in both tobacco leaf and culture cell samples.

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IX. APPENDICES

Phase I, Relevant results from related CropTech research: Development of a highly effective transgene expression system for plants.

Production and analysis of antigen expressing transgenic plant material for oral delivery has been limited by the expression levels achievable by current methodologies. CropTech's proprietary expression system directly addresses this limitation. We have used this expression system to produce the human lysosomal enzyme glucocerebrosidase, a complex glycoprotein with significant value as replacement enzyme for Gaucher disease. We have generated multiple transgenic tobacco plants that produce the human enzyme in active form at levels exceeding 10% of soluble protein in transgenic tobacco leaves. Our expression strategy is quite novel - little or no expression of the foreign transgene is evident during normal growth and development of the transgenic plants (or during growth of suspension-cultured cells in absence of inducer). Leaves are harvested, removed to the processing facility, and induced under controlled conditions to activate the engineered transgene. For the human glucocerebrosidase, protein is extracted eight to twelve hours following induction. This strategy has several key advantages for specialized protein production. All proteins are newly synthesized avoiding any "presentation" of the antigen in the field or greenhouse and wasteful synthesis/degradation cycles common using constitutive expression strategies. In addition, the induction scheme directs very strong transgene activation in all treated tissue. A key issue to be addressed in Phase II is how to utilize this highly effective inducible system with a plant species, tissue, and induction/preparation scheme compatible with and appealing for human consumption. We propose to target tomato for oral antigen delivery because: 1) the fruit is commonly consumed raw (heating is likely to denature proteins and alter antigenicity), 2) *Agrobacterium*-mediated transformation is relatively easy although not as efficient as tobacco. and 3) the CropTech expression system shows high level inducible expression in tomato fruit at all developmental stages tested (Weissenborn and

Cramer, unpublished results). In Phase II, development of a tomato-based system for edible vaccine delivery will be carried out in parallel with experiments testing the efficiency and efficacy of transgenic tobacco to produce bubonic plague antigens that direct oral immunization.

X. PUBLICATION AND PERSONNEL

Publications: None

Key Personnel:

Principal Investigator, Dr. Karen Oishi has wide experience in all aspects of the proposed research. She will direct all personnel on this project and is responsible for overall conduct of this project.

Senior Associate, Dr. Deborah Weissenborn is an expert in transformation and regeneration of tobacco and will conduct this part of the research.

Research Associate, Dr. Quiang Chen, is the major researcher on this project. He is an expert in molecular biology and protein biochemistry.

Research Technician. Mathew Gibney IV had the major responsibility to help with genetic engineering of plants as well as growth and maintainence of transgenic plant material.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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